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The propeptide Asn[1]–Tyr[126] is the storage form of rat atrial natriuretic factor

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Granules from rat atria were isolated by differential centrifugation and by a 53% (v/v) Percoll gradient after tissue homogenization in 0.25 M-sucrose/50 mM-Na_2EDTA. About 40% of the immunoreactive ANF (atrial natriuretic factor) sedimented with the atrial granules during differential centrifugations. On the Percoll gradient, two distinct bands were observed. Cell debris, mitochondria, lysosomes, myofilaments and microsomes were mostly contained in the lightest-density (ρ) (1.03–1.07 g/ml) fraction, as demonstrated by electron microscopy and by enzymic markers such as lactate dehydrogenase, monoamine oxidase, cytochrome *c* reductase, β-glucuronidase and acid phosphatase. Atrial granules were mostly contained in the denser (ρ 1.11–1.15 g/ml) band and were only slightly contaminated by lysosomes, as shown by β-glucuronidase activity. Analysis of the ANF content in these isolated granules by h.p.l.c., amino acid composition and sequencing demonstrated that it was only the pro-ANF [ANF-(Asn[1]–Tyr[126])-peptide]. The precursor was present in all granules, as demonstrated by immunocytochemistry. Since hormonal propeptides usually undergo intracellular processing, and the matured peptides are subsequently stored in the secretory granules, these results indicate that the processing pathway of ANF may be different from that of other hormonal peptides.

INTRODUCTION

The presence of dense granules in mammalian atrial cardiocytes has been described more than 25 years ago by Kisch [1] and by Bompiani *et al.* [2]. It was suggested that these atrial granules were the intracellular storage sites of catecholamines, since reserpine caused a decrease in their number [3]. It was later shown that the extent of granulation [4,5] was affected by changes in water or sodium intake. The atrial granules display properties similar to those of secretory granules containing polypeptide hormones: the presence of a perigranular membrane, intracellular localization and the kinetics of incorporation of labelled amino acids or sugar [6].

de Bold *et al.* [7] reported the presence of a natriuretic factor in the atria. This factor was closely associated with the granules, as demonstrated by co-sedimentation [8,9] or by immunocytochemistry [10]. The atrial natriuretic factor (ANF), which is now well characterized (for reviews see [11] and [12]), is found in the circulation as a peptide of 28 amino acids [ANF-(Ser[99]–Tyr[126])-peptide] [13,14]. However, in the atria, as well as in cardiocytes in culture, ANF is present as a 15–17 kDa peptide [15–17]. Biosynthetic studies employing ^{35}S-labelled amino acids have demonstrated that this large peptide is also released unaltered into the culture medium [17]. However, the exact nature of intracellular ANF has not yet been clearly demonstrated.

In order to investigate the intracellular storage form of ANF, the secretory granules from rat atria were isolated, ANF was purified and its amino acid sequence determined.

MATERIALS AND METHODS

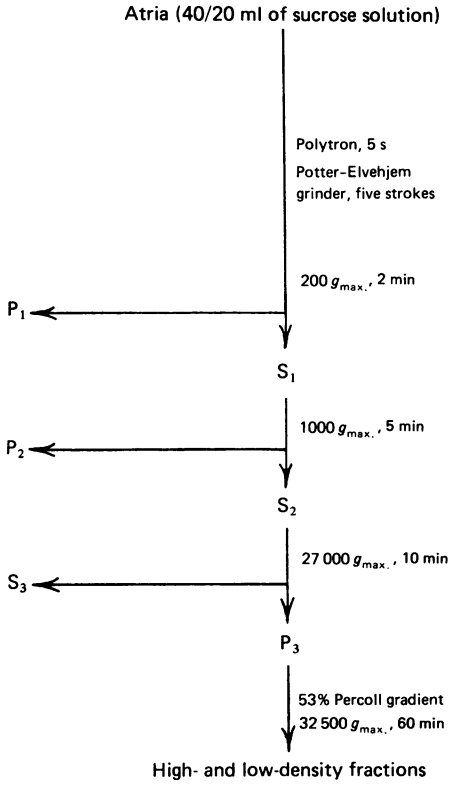
Materials

ANF-(Arg[101]–Tyr[126])-peptide and *N*-terminal fragments of ANF [ANF-(Asp[11]–Ala[37])-, -(His[21]–Ala[37])-, and (Pro[57]–Leu[72])-peptides] were purchased (Institut Armand Frappier, Laval, Canada). Percoll, bead density markers and Protein A were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Substrates for enzymic markers, such as NADH, cytochrome *c*, 4-methylumbelliferyl β-D-glucuronide, *p*-nitrophenyl phosphate, as well as fluorescamine, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [^{3}H]Tryptamine (39.6 Ci/mmol) came from New England Nuclear Corp., Boston, MA, U.S.A. Araldite was purchased from Ladd Research Industries, Burlington, VT, U.S.A. Lowicryl K_4M came from Polysciences, Warrington, PA, U.S.A.

Preparation of atrial granules

Atrial appendages of 20 female adult Sprague–Dawley rats (200–250 g body wt.) were dissected and placed in 0.25 M-sucrose containing 10 mM-Tris/HCl and 50 mM-Na_2EDTA, pH 7.4 (hereafter called 'sucrose solution'). All the following steps (Scheme 1) were performed at 4 °C. The atria were rapidly washed in the sucrose solution to remove blood, minced with a scalpel and then homogenized in the same solution (two atria/ml) in a Polytron homogenizer for 5 s at maximum power and then with a Potter–Elvehjem grinder [0.13 mm (0.005 in) clearance] for five strokes at 700 rev./min. The homogenate was first centrifuged at 200 $g_{max.}$ for 2 min to yield the P_1 pellet. All centrifugations were performed

Abbreviations used: ANF, atrial natriuretic factor; IR-ANF, immunoreactive atrial natriuretic factor; PTH, phenylthiohydantoin.



Scheme 1. Isolation of atrial granules by differential centrifugation

in a Sorvall SS-34 rotor at 4 °C. The supernatant (S_1) was then centrifuged at 1000 $g_{max.}$ for 5 min (P_2 pellet). The resulting supernatant (S_2) was further re-centrifuged at 27000 $g_{max.}$ for 10 min to yield the crude granule fraction (P_3 pellet). The pellet was gently dispersed in 35 ml of 53% Percoll containing 0.25 M-sucrose, 25 mM-Na_2-EDTA and 10 M-Tris/HCl, pH 7.4. The Percoll gradient was initiated by centrifugation at 32500 $g_{max.}$ for 60 min. The fractions of the Percoll gradient were aspirated from the bottom of the tube with a peristaltic pump. A flow chart of this procedure is given in Scheme 1.

Analytical procedures

The density of the Percoll gradient was measured by using density marker beads. Proteins were quantified with fluorescamine as described by Yokosawa *et al.* [18]

The following enzymic markers were analysed: lysosomes: acid phosphatase and β-glucuronidase activities with *p*-nitrophenyl phosphate [19] and with 4-methylumbelliferyl β-D-glucuronide [20] as substrates respectively; cytosol: lactate dehydrogenase with pyruvate as substrate [21]; endoplasmic reticulum: NADH:cytochrome reductase with cytochrome as substrate [22]; mitochondria: monoamine oxidase with [^{3}H]tryptamine as substrate [23].

The radioimmunoassay of ANF was performed with rat ^{125}I-ANF-(Arg^{101}–Tyr^{126})-peptide as a tracer, and with a rabbit antiserum against rat ANF-(Arg^{101}–Tyr^{126})-peptide [24]. Briefly, ANF standards [100 μl, 3–1560 pg of ANF-(Arg^{101}–Tyr^{126})-peptide] or samples were incubated overnight at 4 °C with 8000 c.p.m. of ^{125}I–ANF–(Arg^{101}–Tyr^{126})-peptide, 100 μl of rabbit antiserum (diluted 1:50000) and 100 μl of 0.1 M-sodium phosphate buffer (pH 7.4)/1% (w/v) bovine serum albumin/0.05 M-NaCl/0.1% Triton X-100/0.01% NaN_3. Separation of antibody-bound ANF was performed by addition of 100 μl of goat anti-rabbit immunoglobulin (diluted 1:50), and 100 μl of rabbit normal serum (diluted 1:35). After incubation for 2 h at room temperature, 1 ml of 6.25% (w/v) poly(ethylene glycol) 8000 was added to each tube. They were then centrifuged at 2000 $g_{max.}$ for 20 min and the pellets counted for radioactivity. The ANF concentration which displaced iodinated ANF by 20% was 19.6±9.9 pg/tube. The intra- and inter-assay variations were lower than 15%. The antibody, which is directed against the disulphide loop, demonstrated high cross-reactivity with short forms of ANF, but only 20–50% with longer forms [24].

Isolation and analysis of pro-ANF

Fractions 2–5 of the Percoll gradient, which contain granules and IR-ANF, were pooled, and acetic acid was added to a final concentration of 15% (v/v). Percoll beads were removed by centrifugation at 40000 $g_{max.}$ for 10 min, the pellet washed with 0.1% (v/v) trifluoroacetic acid and re-centrifuged at the same speed. The supernatants were pooled and injected on to a C_{18} μ-Bondapak column (0.78 cm × 30 cm). The material was eluted with a linear gradient of 15–55% (v/v) of acetonitrile, containing 0.1% trifluoroacetic acid, with a slope of 0.5%/min and a flow of 2 ml/min; 2 min fractions were collected.

The purity of the immunoreactive peak was further assessed on a C_{18} Vydac column (0.30 cm × 25 cm) with a linear gradient of 25–50% acetonitrile, containing 0.1% trifluoroacetic acid, with a slope of 0.33%/min and a flow rate of 1 ml/min.

The *N*-terminal sequence characterization of 25 μg of the purified peptide was accomplished by automated gas-phase sequencing performed on an Applied Biosystems model 470A protein sequencer. The glass-fibre filter was loaded with 30 μl of a Biobrene solution (corresponding to 3 mg of Biobrene in 0.1 M-NaCl; Applied Biosystems) and precycled for four cycles according to the manufacturer's protocol. The h.p.l.c.-purified fraction was then loaded by using multiple addition of 30 μl aliquots. All reagents and solvents were obtained from Applied Biosystems and the sequencer was run according to the manufacturer's instructions. After conversion with 25% (v/v) trifluoroacetic acid at 50 °C, the PTH derivatives were transferred from the conversion flask with a mixture of methanol/acetonitrile (1:1, v/v) directly into the Wisp insert vial (Waters, Milford, MA, U.S.A.) and evaporated to dryness in the presence of 500 pmol of PTH-norleucine, acting as an internal standard, in a Speed Vac apparatus (Savant Instruments). The dried samples were reconstituted first by adding 20 μl of acetonitrile, vortex-mixing and then adding 80 μl of water; 80% of the material was analysed directly by reversed-phase h.p.l.c. as previously described [25].

For the amino acid composition, two aliquots of the h.p.l.c.-purified fraction were freeze-dried in the presence of 2.5 nmol of norleucine. Amino acid analysis was thus performed in duplicate after hydrolysis in 5.7 M-HCl containing a trace of phenol and 0.01% (v/v) mercaptoethanol *in vacuo* at 108 °C for 24 h. The separation and quantification of the amino acids were done by using a Beckman 120C instrument equipped with a model 126 computing integrator.

Electron microscopy of the Percoll fraction

To 1 ml of the Percoll fractions was added 3 ml of 2% (w/v) glutaraldehyde in cacodylate/HCl, 0.1 M, pH 7.1. After 1 h at room temperature the solutions were centrifuged at 100000 $g_{max.}$ for 60 min. The pellet was rinsed in cacodylate buffer, post-fixed in 2% (w/v) OsO_4 buffered with Veronal acetate, dehydrated and embedded in Araldite. Fine sections were cut on a Reichert ultramicrotome (Omu2) and stained with uranyl acetate and lead citrate. They were examined in a JEOL 1200EX electron microscope.

Immunocytochemistry

The right and left atria of control female Sprague–Dawley rats (190–210 g) were fixed with 1% (w/v) glutaraldehyde buffered with cacodylate/HCl (0.1 M, pH 7.1) perfused simultaneously through the right and left cardiac ventricles for 10 min. Fragments of atria were then rinsed, fixed for a further 1 h in the same manner and placed in cacodylate buffer containing 2% (w/v) sucrose at 4 °C for 24 h. The specimens were embedded in Lowicryl K_4M at −45 °C [26].

Colloidal gold was prepared to obtain small (12.69 ± 0.16 nm) particles [26]. The gold suspension was then boiled for an additional 15 min, adjusted to pH 6.9 with 0.2 M-K_2CO_3 and used to label Protein A as described by Roth *et al.* [27].

The nickel grids with mounted fine sections were incubated on a drop of phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.4) containing 1% (w/v) ovalbumin for 5 min. The grids were transferred on a drop of antiserum diluted 1:20 and incubated for 60 min at room temperature. The antibodies were produced in rabbit after subcutaneous injections of ANF-(Asp^{11}–Ala^{37})-, -(His^{21}–Ala^{37})- and -(Pro^{57}–Leu^{72})-peptides which had been previously coupled to thyroglobulin as described by Gutkowska *et al.* [24]. After a rapid rinse in phosphate-buffered saline, the grids were incubated with the Protein A–gold complex for 30 min at room temperature, thoroughly washed, rinsed in distilled water and dried. The sections were then stained with uranyl acetate alone or in combination with lead citrate and examined with a JEOL 1200EX electron microscope. The specificity of immunostaining was assessed with the following controls: (1) incubation of the thin sections with antiserum previously absorbed with the corresponding peptide; (2) incubation of the thin sections with antiserum, followed by 1 h incubation with unlabelled Protein A (0.2 mg/ml) and then with the Protein A–gold solution; (3) omission of the antiserum step and application of the Protein A–gold solution alone. All these controls gave negative results, i.e. insignificant random deposits of gold particles on the sections.

RESULTS

Isolation of atrial granules

Rat atria were homogenized in a sucrose solution containing 50 mM-Na_2EDTA. Homogenization of the atria with an EDTA concentration lower than 10 mM results in the presence of 80–90% of immunoreactive ANF in the P_1 pellet. A concentration of EDTA higher than 25 mM was thus used to separate atrial granules from other organelles. Table 1 gives the distribution of proteins, IR-ANF and lysosomal markers in the fractions obtained by differential centrifugation. β-Glucuronidase activity essentially paralleled the distribution of IR-ANF, which means that the lysosomes were also pelletted simultaneously with the atrial granules. The acid phosphatase activity, however, was dissociated from β-glucuronidase, and more than 50% remained in the S_3 supernatant. Acid phosphatase may not be a good marker for lysosomes in the atrial tissue. As compared with the proteins, a 2-fold enrichment in IR-ANF was obtained in P_3.

In order to separate granules from lysosomes and other cell debris, the P_3 pellet was submitted to a 53% Percoll gradient. After centrifugation, two bands were clearly observed in the centrifugation tubes. The first one, on the top of the gradient, appeared light brown. The second, at the bottom, appeared darker and more diffuse.

Analysis of fractions, taken from the bottom of the tubes, is illustrated in Fig. 1. The first band, which had the lowest density (ρ = 1.03–1.07 g/ml) contains large quantities of protein, β-glucuronidase, acid phosphatase, monoamine oxidase, lactate dehydrogenase and cytochrome *c* reductase activities (Table 2). The band at the bottom of the gradient (ρ = 1.11–1.15 g/ml) contains about 60% of the IR-ANF. This band was also partially associated with β-glucuronidase activity. Analysis of other marker enzymes reveals only minor contamination of this band. As compared with the protein content of this region, an overall purification of 8-fold of IR-ANF was obtained.

Electron microscopy of the two regions, after fixation by glutaraldehyde, is shown in Fig. 2. The band with the lowest density (Fig. 2*a*) was composed of cell debris,

Table 1. Distribution of protein, IR-ANF and enzymic markers in fractions obtained by differential centrifugation of rat atria homogenates

Rat atria were homogenized in sucrose solution and submitted to differential centrifugation as shown in Scheme 1. Portions of the different pellets and supernatants were kept in order to measure IR-ANF, proteins and enzymes as described in the Materials and methods section. Total activities (100%) were considered as the sum of P_1, P_2, P_3 and S_3.

Protein or marker	Fraction ...	Distribution (%) (mean ± S.E.M.)					
		P_1	P_2	P_3	S_1	S_2	S_3
Protein (n = 4)		18.4 ± 5	8.6 ± 3.4	24.8 ± 1.7	60.8 ± 5.7	58.9 ± 4.6	48.3 ± 6.2
β-Glucuronidase (n = 3)		19.4 ± 0.6	10.2 ± 3.1	33.1 ± 1.8	70.4 ± 4.1	56.3 ± 9.5	37.2 ± 1.7
Acid phosphatase (n = 3)		23.7 ± 3.0	7.6 ± 2.1	14.0 ± 0.5	72.2 ± 7.1	64.7 ± 7.8	54.7 ± 4.7
IR-ANF (n = 5)		28.5 ± 6.0	7.4 ± 2.7	40.8 ± 7.4	84.6 ± 18.5	69.9 ± 22.1	23.2 ± 4.2

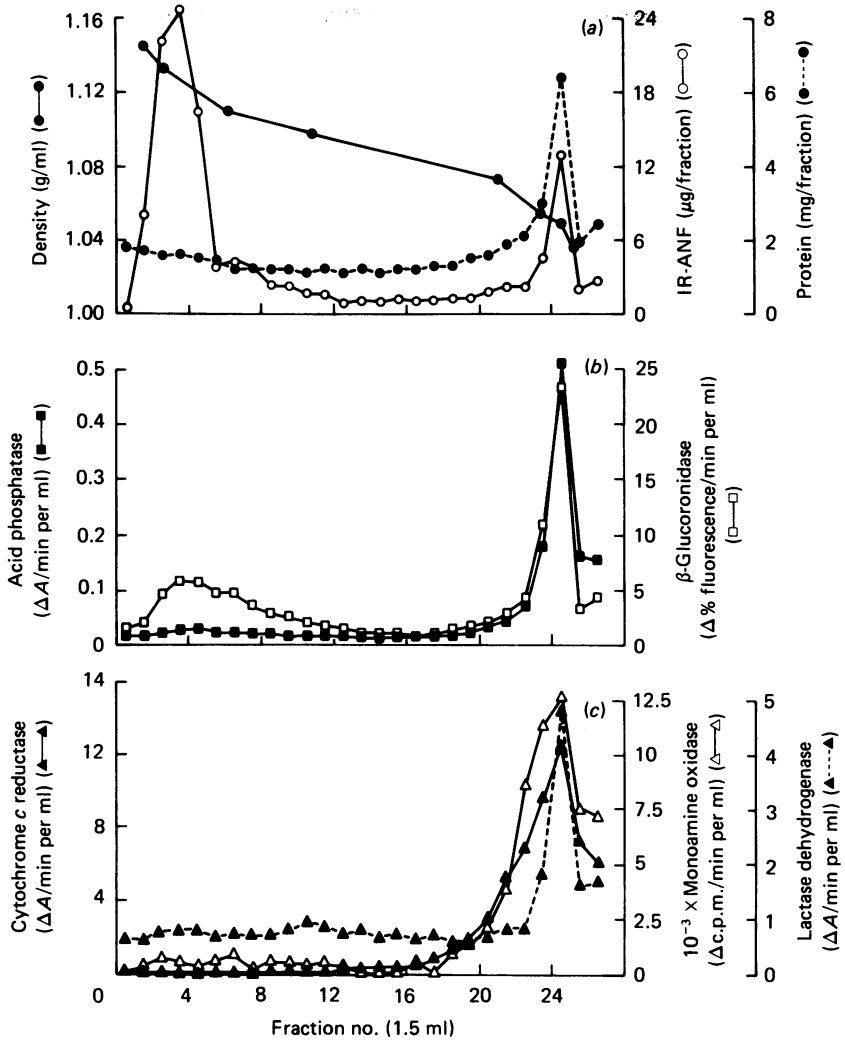


Fig. 1. Typical distribution of protein, IR-ANF and enzymic markers in the fractions from the 53% Percoll gradient

Fractions (1.5 ml) were collected from the bottom of the centrifugation tube. In each fraction, we measured IR-ANF, proteins and enzymes as described in the Materials and methods section. (*a*) Density, protein and IR-ANF; (*b*) lysosomal markers (β-glucuronidase and acid phosphatase); (*c*) cytochrome *c* reductase (marker of the endoplasmic reticulum), monoamine oxidase (mitochondrial marker) and lactate dehydrogenase (marker of cytosol).

mitochondria, lysosomes, myofilaments, microsomes and a few granules. The band of higher density (Fig. 2*b*) was mainly composed of atrial granules contaminated with few lysosomes and, rarely, mitochondria. The maximum diameter of the granules was 375 nm, which is within the range of the values already reported [28].

Identification of the granule content

Fractions of the Percoll gradient with a density between 1.11 and 1.15 g/ml were pooled and acetic acid added in order to destroy proteinases. The material obtained from 20 rats was then separated by h.p.l.c. on a semipreparative C_{18} μ-Bondapak column (Fig. 3). A major absorbance peak eluted between 58 and 60 min at 42% (v/v) acetonitrile contained all the IR-ANF. No immunoreactivity was found to be eluted between 25 and 30% acetonitrile contained all the IR-ANF. No immunoreactivity was found to be eluted between 25 and 30% Vydac column only slightly improved its purity.

Analysis for 25 cycles of the *N*-terminal amino acid

Table 2. Distribution of proteins, IR-ANF and enzymic markers in fractions obtained from the Percoll gradient

Data from different experiments, such as in Fig 1, and corresponding to densities of 1.11–1.15 g/ml and 1.03–1.07 g/ml were added up and expressed as percentages of the total.

Protein or marker	ρ (g/ml) ...	Distribution (%) mean ± S.E.M. 1.11–1.15	1.03–1.07
IR-ANF ($n = 6$)		57.3 ± 5.5	19.3 ± 4.3
Proteins ($n = 5$)		12.1 ± 2.9	51.5 ± 15.6
β-Glucuronidase ($n = 7$)		17.3 ± 6.7	61.9 ± 9.0
Acid phosphatase ($n = 3$)		3.1 ± 1.5	89.6 ± 2.8
Monoamine oxidase ($n = 3$)		4.1 ± 2.6	74.5 ± 9.3
Lactate dehydrogenase ($n = 3$)		6.1 ± 1.1	60.0 ± 11.1
Cytochrome *c* reductase ($n = 3$)		0.3 ± 0.1	88.1 ± 8.2

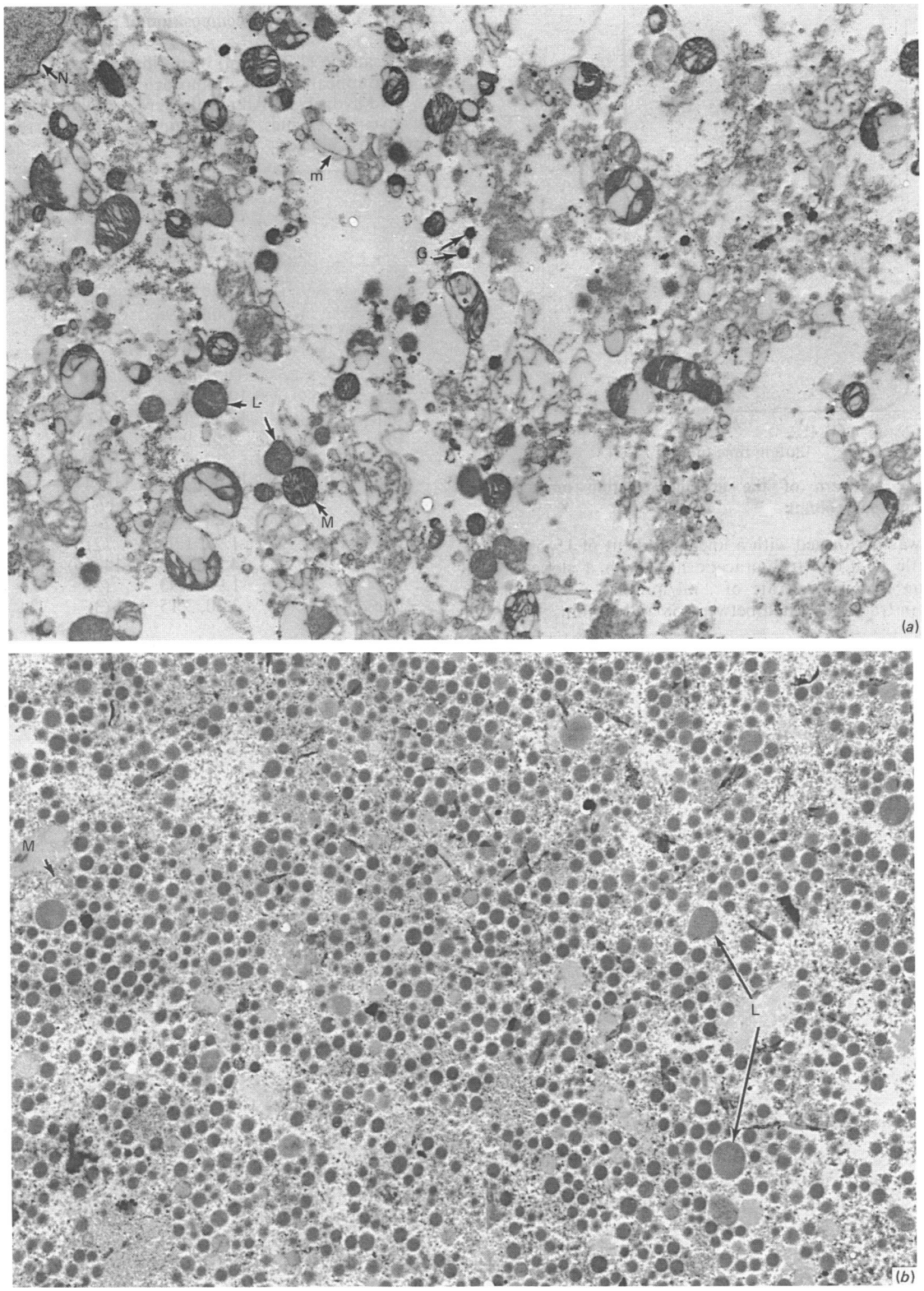


Fig. 2. Electron micrograph of the fractions from the 53% Percoll gradient

(*a*) Electron micrograph of the light-density (1.03–1.07 g/ml) fraction after fixation with 2% glutaraldehyde. Abbreviations used: N, nucleus; M, mitochondria; m, microsome; G, granules; L, lysosome. Magnification × 6800. (*b*) Electron micrograph of the high-density (1.11–1.15 g/ml) fraction after fixation with glutaraldehyde. 'M' and 'L' were defined in (*a*). Magnification × 6800.

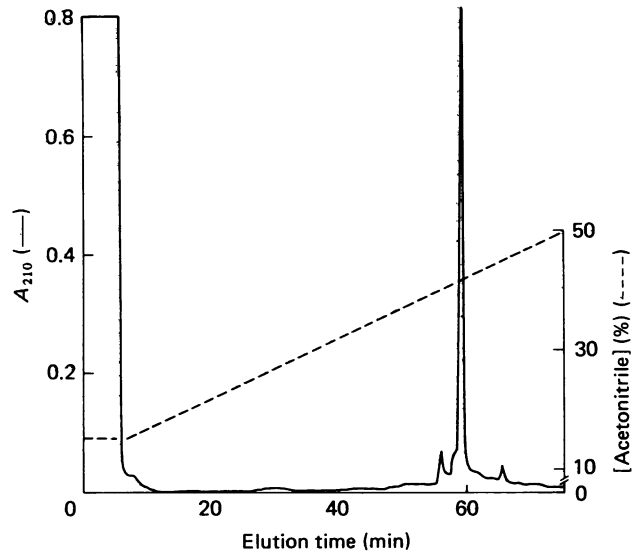


Fig. 3. H.p.l.c. pattern of the granule fraction on a C_{18} μ-Bondapak column

Elution was performed with a linear gradient of 15–50% acetonitrile in 0.1% trifluoracetic acid with a slope of 0.5%/min and a flow rate of 2 ml/min. IR-ANF was detected in fractions eluted between 58 and 60 min.

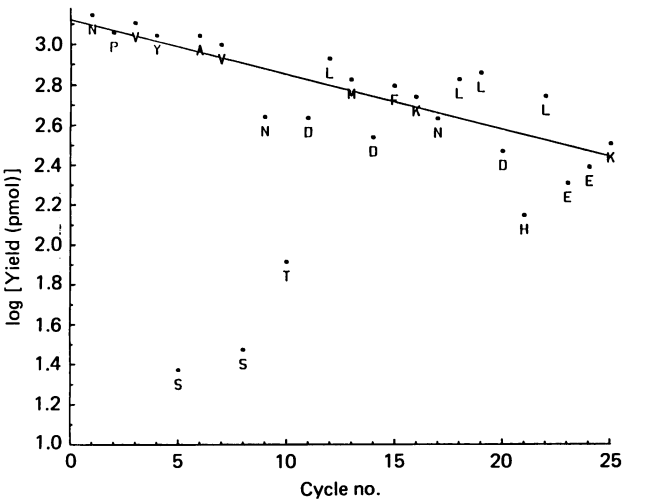


Fig. 4. *N*-Terminal amino acid sequencing of IR-ANF

The peptide (25 μg) was submitted for 25 cycles to Edman degradation on a gas-phase amino acid sequencer. The repetitive yield was 93.9% on the basis of selected stable PTH-amino acids. The one-letter code for amino acids is used.

sequence of 25 μg of this peptide reveals a perfect homology with the first 25 residues of the pro-ANF, which contains 126 amino acids (Fig. 4). The amino acid composition of 17 μg of the peptide, as shown in Table 3, demonstrates the presence of two tyrosine and ten arginine residues. These data indicate that the last *C*-terminal amino acid is probably tyrosine-126, which is not followed by the two arginine residues found in the DNA sequence of the rat gene [29]. This peptide was therefore identified as ANF-(Asn^{1}–Tyr^{126})-peptide.

Table 3. Amino acid composition of purified pro-ANF

A sample (1.25 nmol) of purified pro-ANF was analysed in duplicate for its amino acid composition after a 24 h acid hydrolysis. All results were computed by assuming 12 glycine residues. Values in parentheses represent the nearest integer.

	Yield (residues/molecule)		
	Obtained		Theoretical
Lys	3.87, 3.78	(4)	4
His	1.06, 0.96	(1)	1
Arg	9.60, 9.58	(10)	10
Asp	13.40, 13.54	(13–14)	14
Thr	3.21, 3.24	(3)	3
Ser	12.96, 13.31	(13)†	15
Glu	12.41, 12.37	(12)	12
Pro	9.65, 9.44	(10)	10
Gly	12, 12	(12)	12
Ala	10.60, 10.52	(10–11)	10
Val	7.14, 6.76	(7)‡	6
Met	2.44, 2.36	(2–3)	3
Ile	1.96, 1.91	(2)	7
Leu	14.30, 14.52	(14–15)	15
Tyr	1.96, 1.93	(2)	2
Phe	3.10, 3.15	(3)	3
Cys*	–		2
Trp*	–		2

* Tryptophan and cysteine were not quantified.
† A 10–15% loss was not considered.
‡ Overestimated due to buffer change.

Furthermore, on the basis of the yield of amino acids, more than 100 μg of pure pro-ANF was isolated from the atrial granules of 20 rats.

Immunocytochemistry of the atrial cardiocytes (Fig. 5) using antisera directed against different parts of pro-ANF, namely residues 11–37, 21–37 and 54–72, indicated that all specific granules of both atria, either of the A, B or D type [1,2], were reactive. The gold particles were not present in any other structure in any significant numbers.

DISCUSSION

ANF was found to be localized in specific granules as demonstrated by bioassay [8,9] and by immunocytochemistry [10]. de Bold *et al.* first described isolation of atrial granules by differential centrifugation followed by a discontinous sucrose gradient [8,30]. In our hands this procedure was often not reproducible, probably owing to the low concentration of EDTA used. We improved the procedure and followed the isolation of the granules with the measurement of IR-ANF. IR-ANF was closely associated with atrial granules, confirming that they are the site of storage of ANF. Characterization of the granules, by enzymic markers demonstrated that they were not associated with lactate dehydrogenase, monoamine oxidase, cytochrome *c* reductase and acid phosphatase. However, the β-glucuronidase activity was partially co-purified with the granules, indicating slight contamination by high-density lysosomes. The development of this procedure for the isolation of pure atrial granules will thus permit one to study the biochemical

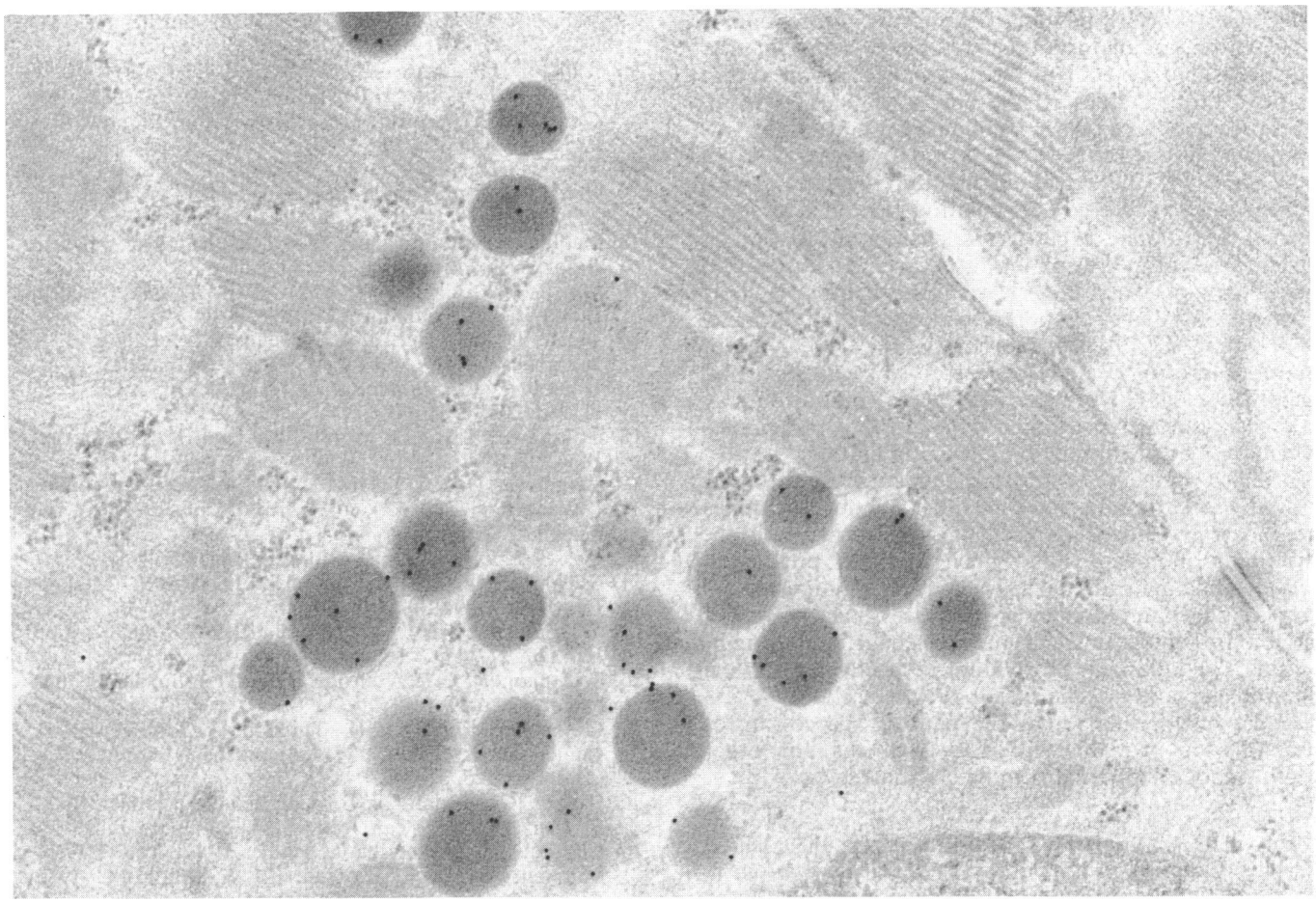


Fig. 5. Electron micrograph of atrial cardiocytes incubated with antisera against *N*-terminal fragments of ANF

The tissue was first incubated with rabbit antiserum against an ANF fragment [ANF-(Asp^{11}–Ala^{37})-peptide] and then with electron-dense Protein A–gold particles. Identical results were obtained with antibodies against the other peptides [ANF-(His^{21}–Ala^{37})- and ANF-(Pro^{57}–Leu^{72})-peptide]. Dense particles were mainly localized on the atrial granules. Magnification × 48400.

components of these organelles such as ANF or possible maturation enzyme(s).

Purification of ANF from these granules and its identification by amino acid composition and sequencing revealed that it corresponds to the propeptide ANF-(Asn^{1}–Tyr-^{126})-peptide, and no significant amount of short forms, such as ANF-(SER^{99}–Tyr^{126})-peptide, can be detected. Interestingly, as indicated by the h.p.l.c. pattern (Fig. 3), this peptide appears to represent the major protein component of the granules, although many other proteins may have precipitated in the presence of 15% acetic acid. Under these conditions, short forms of ANF are stable and, in fact, acetic acid has recently been used to isolate ANF from atrial extracts [12] or to extract it from plasma [31]. Furthermore, atrial cells, when carefully homogenized in acetic acid, contain minimal amounts of short ANF forms [16]. We consistently obtained about 50–75 μg of IR-ANF (100–150 μg of peptide on the basis of amino acid analysis) from each purification batch; 100 μg of the propeptide can thus be easily obtained in a single day by isolation of the granules and by one reversed-phase h.p.l.c. purification step. The availability of this peptide will permit further biochemical studies of ANF such as its degradation in blood or its maturation.

Immunocytochemistry using antibodies against the *N*-terminal portion of the molecule indicates that the precursor, or part of it, was at least present in all types of granules. Morphological differences among the granules may therefore be due to their condensation state rather than to the nature of ANF itself. Similar results were obtained with antibodies against the *C*-terminal part of ANF [10].

It is noteworthy that the precursor does not contain two arginine residues in positions 127 and 128, which are encoded by the DNA sequence of the gene [29]. The prepropeptide may be processed during the early post-translational steps, which would remove the basic residues by a carboxypeptidase-B-like converting enzyme. This type of enzyme has been found in secretory granules of different tissues [32].

These results confirm reports indicating that the atrial tissue or cardiocytes in culture contain a high-M_r form of ANF [15–17]. Furthermore, we have clearly elucidated the primary structure of its storage form. Peptidic prohormones usually undergo post-translational modifications in the Golgi complex and in the secretory granules, which lead to the subsequent secretion of mature hormones [33]. Since the storage form of ANF is the precursor and the major circulating form is

ANF-(Ser^{99}–Tyr^{126})-peptide, the next question is whether or not maturation takes place intracellularly. Glembotski *et al.* [16] and Bloch *et al.* [17] reported that cultures of atrial cells release only a 15–17 kDa form. This precursor form, when incubated with rat serum, was converted into a 3 kDa ANF form. However, rat serum may not be the ideal incubation medium, since proteinases of the blood coagulation cascade have been activated. Different experiments, such as incubation of atrial slices or perfusion of rat hearts, in absence of blood, demonstrated the release in the media of a short form of ANF that corresponds to its circulating form, i.e. ANF-(Ser^{99}–Tyr^{126})-peptide [15,31,34]. The site of maturation of ANF therefore remains obscure, since no definitive result has yet been presented. Maturation may take place during secretion or in the blood by proteinases which may be membrane-bound or circulating.

The fact that ANF is stored as a precursor in the atrial granules is intriguing, since all other peptide hormones are generally stored in their mature form, with the exception of enkephalins, which are also present as pro-enkephalin in adrenal cells [35, 36]. Angiotensinogen and kininogen in blood are special cases, since renin and angiotensin-converting enzyme, which generate angiotensin I and destroy bradykinin respectively, are the limiting step and not the precursors themselves.

These results imply that the pathway processing of pro-ANF is probably different from that of other prohormones and remains to be elucidated.

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